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Heterogeneity of Mitochondrial DNA Haplotypes in Pre-Columbian Natives of the Amazon Region

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ABSTRACT We report the first study of mitochondrial DNA (mtDNA) sequencing from ancestral Amerindian populations of the South American continent. Sequencing of the D-loop region of mtDNA was carried out for bone fragments from 18 skeletons of Pre-Columbian Amerinidians. The skeletons were excavated in different archeological sites of the Brazilian Amazon region, with dating estimated at 500–4,000 years before the present. The sequencing of at least 354 bases permitted the identification of 13 haplotypes defined by variation of 26 nucleotide positions. Two haplotypes were shared by more than one sample, while 11 haplotypes were observed for a single sample. Seven haplotypes observed in 11 individuals (61% of the sample) belong to the four haplogroups described by Horai et al. (1993). Three samples that shared the transition C → T in positions 16,223 and 16,278 formed a fifth haplogroup, which has been previously described in present-day Indian populations. Finally, four samples formed a heterogeneous group but each haplotype had at least one mutation typically detected in Asian or Mongoloid populations. Thus, although only haplotypes shared by Asian populations were detected, a wide haplotype variability was observed. If our sample is representative of Pre-Columbian South America, the percentage of haplotypes (39%) not belonging to the four haplogroups described by Horai is much greater than in contemporary indigenous populations. This permits us to suggest that, in addition to the postulated bottleneck effect during the migration from Asia to the Americas, the depopulation effect started by European colonization in the 16th century contributed to the reduction in genetic variability of Amerindians. © 1996 Wiley-Liss, Inc.

Mitochondrial DNA (mtDNA) has been used extensively as a tool for the reconstruction of the human evolutionary history because of its maternal inheritance, lack of recombination, and high mutation rate relative to nuclear DNA (Giles et al., 1980). A single cell may have as many as 3×10^3 identical copies of its genome, a fact that makes the mitochondrial genome quite accessible, especially in studies of populations that lived in the past and whose tissues were partially preserved.

Several investigators have studied the mtDNA of present-day indigenous American populations in an attempt to answer questions about their genetic diversity, relationship to other human groups, migration times, and bottleneck effects. There is no agreement about data interpretation, and

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even the possibility of a restricted number of ancestral lineages has been questioned. The study of conserved biological material in bones and skin may help resolve these questions.

Studies of ancient mtDNA (Pääbo, 1989) convey important information from the archaeological and anthropological viewpoint. In a study of prehistoric material, Hagelberg's group suggested that the first inhabitants of Polynesia were descendants of people from Melanesia (Hagelberg and Clegg, 1993) and that the first colonizers of Easter Island were of Polynesian origin (Hagelberg et al., 1994).

Stone and Stoneking (1993) studied samples of 50 Pre-Columbian skeletons from the Norris Farm cemetery, Illinois, dated to 1300 AD. Their results demonstrated that 98% of the mtDNA types detected in this prehistoric population belong to one of the four founding lineages that may have given origin to all present-day Amerindians (Schurr et al., 1990; Wallace and Torroni, 1992; Torroni et al., 1993a,b, 1994). Horai et al. (1991) found the 9 bp deletion between COII and $tRNA^{Lys}$ genes in only one of 11 mummies from the southeast USA and northern Chile. Two other mummies from South America have been studied by Monsalve et al. (1994) using RFLP of mtDNA: they amplified a 478 bp DNA segment and showed that it differed from the haplotypes observed in 31 contemporary South American aborigines but had been previously described in two Mayans (Schurr et al., 1990).

The reduced number of mitochondrial lineages detected among contemporary Amerindians may be the product of a bottleneck effect during migration from Asia to the Americas (Wallace and Torroni, 1992), or the consequence of the drastic reduction in the number of individuals after contact with Europeans (Ubelaker, 1992; Cunha, 1992), or both. In any case, the idea of a reduced number of founding lineages is not consensual (Ward et al., 1991; Horai et al., 1993; Balliet et al., 1994).

In an attempt to answer questions about the initial colonization of the Americas, we sequenced the D-loop region of mtDNA from 18 bone fragments recovered in the Brazilian Amazon region. To our knowledge, this is the first study in which mtDNA sequencing has been used to characterize ancestral populations in the South American continent.

MATERIALS AND METHODS

Specimens

We analyzed the DNA from bone fragments from 26 skeletons which constitute the entire collection of the Museu Paraense Emílio Goeldi, Pará, Brazil. Analysis was successful for 18 samples, as described in the Results. The skeletons were excavated at 11 different archaeological sites in the Brazilian Amazon region, with dating estimated at 500–4,000 years before the present (BP) in the States of Pará, Amapá, and Amazonas. Table 1 presents an individual list of the samples with their respective excavation sites and estimated ages. In all cases, the bones were from skeletons buried in fetal position in sambaquis or in anthropomorphic urns, and the age of the sample was estimated on the basis of the period to which the pottery belonged.

Special care to avoid contamination with contemporary DNA

Special care must be taken to reduce the possibility of contamination of ancient samples with modern DNA, although it cannot be completed ruled out. Contamination of the outer surface of the bones with human DNA may occur during the unearthing, classification, or storing; contamination in the laboratory may occur during extraction or amplification of the templates for sequencing. The following procedures were employed to avoid or to rule out contamination, most of them suggested by Pääbo (1990): (1) use of gloves and surgical masks during sample manipulation, and blocked pipette tips; (2) the outer bone surface was irradiated with UV light, and the material for the DNA extraction was obtained from the interior spongy region of the bone through a small hole; (3) the extraction method has a small number of steps; (4) DNA extraction was carried out in duplicate for each sample; for each extraction, a "negative" extraction was carried out, using all the reagents except for the bone powder; this "negative" extraction was subsequently submitted to all proce-

TABLE 1. Description of the specimens studied in the present sample

Sample	Excavation site[1]	Type[2]	Dating (years BP[3])
1	Sambaqui Pirabas (PA)	SB	4,000
2	Aterro dos Bichos (PA)	AU	1,900
3	Marajó (PA)	AU	1,900
7	Rio Pau D'Arco (PA)	AU	500
8	PA-AT-59	AU	600
9	Rio Tocantins (PA)	SB	1,000
10	Sambaqui do Furinho (PA)	SB	4,000
11	Sambaqui de Pirabas (PA)	SB	4,000
12	Rio Maracá (AP)	AU	500
13	Sambaqui Cotias (PA)	SB	4,000
14	Sambaqui de Gastropode (PA)	SB	1,600
15	Sambaqui Marco, Maracanã (PA)	SB	4,000
21	Marajó (PA)	AU	1,900
22	Marajó (PA)	AU	1,900
23	Aterro dos Bichos (PA)	AU	1,900
24	Marajó (PA)	AU	1,900
28	Rio Urubu (AM)	AU	1,300
29	Rio Maracá (AP)	AU	500

[1] Corrêa et al., 1971; Evans et al., 1950; Figueiredo, 1965; Guedes, 1897; Simões, 1981; Simões et al., 1987.
[2] SB, sambaqui; AU, anthropomorphic urn.
[3] Before present.

dures as a "negative" control; (5) amplification of the segments for sequencing was carried out in two steps, each of 35 cycles; the second PCR employed 1–5 μl of the product of the first reaction and a primer pair, which was internal in relation to the first PCR; at this step, "negative" controls (with all reagents except for the DNA) were also used; duplicate sequencing for different DNA extracts of the same sample were carried out for most of the samples; sequencing was performed for both strands, and the results were coincident; (6) no ambiguity was observed when reading the sequencing films; (7) the results of sequencing of the mtDNA of the four investigators who manipulated the samples were different from all the 13 haplotypes obtained for the ancient samples; (8) our laboratory is also involved in mtDNA studies of contemporary Amerindian and black populations; only three of the 13 haplotypes obtained for the ancient samples were also observed among the 43 haplotypes thus far obtained for 92 contemporary Amerindians in our laboratory, and none are similar to the haplotypes obtained for blacks.

DNA extraction

The outer surface of the bones was first irradiated with UV light (260 nm) for 10 min at a distance of 20 cm. Using a dentist's burr previously treated with 70% ethanol and 5% sodium hypochlorite, a small orifice was opened in the bone surface and the material obtained during drilling was discarded. One gram of the spongy layer in powder form was then removed and utilized for DNA extraction. This procedure has two advantages: (1) prevention of contamination with exogenous DNA due to previous sample handling, and (2) preserving as much as possible the integrity of the bone collections.

One part of the powder was mixed with three parts of a buffer containing 10 mM Tris-HCl, 0.1 mM EDTA, 0.01% Triton X-100, and 0.5 mM dithiothreitol, pH 8.0, which was left at room temperature for 2 hr and then heated on a waterbath at 94°C for 30 min, with care taken to shake the preparation at 3 min intervals. After incubation the suspension was centrifuged at 3,000 rpm for 5 min, and the supernatant was removed and transferred to another tube, to which 10% (v/v) 3 M sodium acetate and 2.5 parts absolute ethanol to one part of the supernatant were added. The mixture was kept at −70°C for 6 hr and then centrifuged at 15,000 rpm for 10 min; the supernatant was discarded and the sediment containing DNA was washed with 70% ethanol, dried, and resuspended in 100 μl water for later use in the polymerase chain reaction (PCR).

Polymerase chain reaction (PCR)

The segments under study were amplified using a pair of specific primers complemen-

TABLE 2. Primers used for the amplification and sequencing of the D-loop region of mtDNA[1]

Primer pair no.	Coordinates	Amplified products
1	15.920–15.926 16.478–16.498	598 bp
2	15.977–15.997 16.381–16.401	424 bp

[1]Nucleotide bases numbered according to Anderson et al. (1981), in the 5′ → 3′ direction.

tary to the region flanking the target segment. The conditions of the amplification reaction had to be modified slightly in each case in order to obtain maximal specificity and yield. In general, the reaction was carried out on a total volume of 25 μl containing 100 ng DNA, 10 mM Tris-HCl, pH 8.5, 50 mM KCl, 1.5 μM $MgCl_2$, 0.01% gelatin, 100 μM of each dNTP, 0.25 μM of each primer, and 1 U of Taq DNA polymerase, in a Perkin-Elmer-Cetus thermocycler. Thirty-five amplification cycles (94°C for 20 sec, 55°C for 20 sec, and 72°C for 60 sec) were usually carried out. A negative control containing all the components mentioned above except DNA was used both in the DNA extraction phase and in the amplification phase.

Sequencing

Amplification of the D-loop region was performed in three stages: primer pair number 1 (Table 2) amplified a segment of 598 base pairs. A second amplification was then performed using part (1–5 μl) of the product of the first reaction with the number 2 primer pair (Table 2) and applied to a 424 bp product internal to the previous segment. The product of this second amplification was applied to 0.8% agarose gel and then cut out and added to a mixture of 10 mM Tris and 1 mM EDTA for 24 hr. The samples were purified on a Magic Preps column (Promega). A third, asymmetrical, amplification was performed with the number 2 primer pair, which amplified the segment of 424 base pairs. The product of this single-strand amplification was again purified and sequenced with the use of the limiting primer. The procedure was carried out for both strands of the mtDNA. The sequenase sequencing kit (U.S.B.) was used for sequencing with $\alpha(^{35}S)$dATP labeling.

The sequencing products were separated by electrophoresis, on 6% polyacrylamide gels containing 7 mM urea, for 6 hr at 1,600 V. The gel was then fixed in 5% acetic acid and methanol for 30–60 min, dried, and exposed to Kodak XAR film for 12–48 hr. The sequences reported are the results of complementary sequences verified for the two strands.

RESULTS

We were successful in amplifying the mtDNA of 18 out of 26 bone samples from different archaeological sites in the Brazilian Amazon region. This permitted us to complete the direct sequencing of at least 354 base pairs (np 16,047–16,380), in each sample, which were used for comparison of the sequences. The results obtained are presented in Figure 1. The 18 samples code for 13 haplotypes defined by 26 different polymorphic points, two of which (np 16,292 C-T and nt 16,312 A-G) have not been previously described in contemporary or ancestral Amerindian populations. Three haplotypes were shared by more than one sample: M03 (samples 02, 03, 13, and 21), M05 (08 and 15), and M12 (12 and 23), whereas 10 were specific for a single DNA extract.

When comparing the different nucleotide sequences, we detected seven haplotypes (M01–M07) from 11 individuals (samples 02, 03, 08, 11, 13, 15, 21, 22, 24, 28, 29) representing the four mitochondrial lineages (haplogroups) previously identified in studies of contemporary Amerindian populations. These haplotypes were assigned to the four haplogroups on the basis of the presence of at least one of the characteristic mutations of the respective haplogroup. Six haplotypes (M08–M13), however, did not present any of the mutations characterizing the first four haplogroups (I–IV), although they presented modifications commonly detected in Asian populations, and they were classified as groups V and VI.

Haplotype M01 (sample 11) was characterized by the (T → C) transition in nt 16,217 and by mutations in the hypervariable region: two deletions (np 16,182 and nt 16,183), two insertions (np 16,183.3 and nt 16,183.4), and one (T → C) transition in nt 16,189. Haplotype M02 (sample 28) pre-

Haplotype	Sample	11111111112222222223333333 12478888891247999991112246 14292333927398024581295742 CTCCAA--TCTCTCCCCCTTAGTCCT	Haplogroup
M01	11	....--CCC.C.C.......G...T.	I
M02	28	..TT....C..T.....T....C..C	II
M03	02	T..........T..T......A...C	III
M03	03	T..........T..T......A...C	III
M03	13	T..........T..T......A...C	III
M03	21	T..........T..T......A...C	III
M04	24	...........T..T...........	III
M05	15	..................C.......	IV
M05	08	..................C.......	IV
M06	29	...........T......C.......	IV
M07	22	...........T...T.......T..	IV
M08	01	........CT.T.T..T.........	V
M09	09	.........T.T.T..T.........	V
M10	10	T..........T.T............	V
M11	07	TC.........T..............	VI
M12	12	...........T..............	VI
M12	23	...........T..............	VI
M13	14	...................C.....C	VI

Fig. 1. Nucleotide sequences in the D-loop region of 18 ancestral samples of mtDNAs, as comared with the reference sequence of Anderson et al. (1981). The vertical numbers above each column refer to the Anderson sequence numbers in excess of 16,000 (so the first number is 16,111, the second 16,124, and so on). The three positions numbered 183 are in the hypervariable region and correspond, respectively, to 183, 183.3, and 183.4 (Hori et al., 1993). Roman numerals indicate the haplogroups.

sented a (C → T) transition in nt 16,223 and two (T → C) transitions in nt 16,325 and nt 16,362. Haplotypes M03 and M04 (samples 02, 03, 13, 21, and 24) shared transitions in nt 16,290 (C → T) and in nt 16,319 G-A, the latter absent in 24. Haplotypes M05 and M06 (samples 08, 15, and 29) shared transition (T → C) in nt 16,298, and M07 has the transition C → T in nt 16,327.

All haplotypes described above seem to belong to one of four haplotype groups (Horai et al., 1990, 1993; Torroni et al., 1993a,b) described for most of the contemporary Amerindian populations studied: M01 corresponds to group I, M02 to group II, M03 and M04 correspond to group III, and M05, M06, and M07 may correspond to group IV.

Haplotypes M08–M13 did not present any of the mutations that define haplogroups I–IV, although they present mutations that are commonly observed in Asian populations. They were tentatively classified into two groups, V (M08–M10) and VI (M11–M13). The haplotypes of group V (samples 01, 09, and 10) shared transition (C → T) in nt 16,223 and nt 16,278. Haplotypes M11–M13 (samples 07, 12, 14, and 23) did not cluster into a separate group (Fig. 2), and

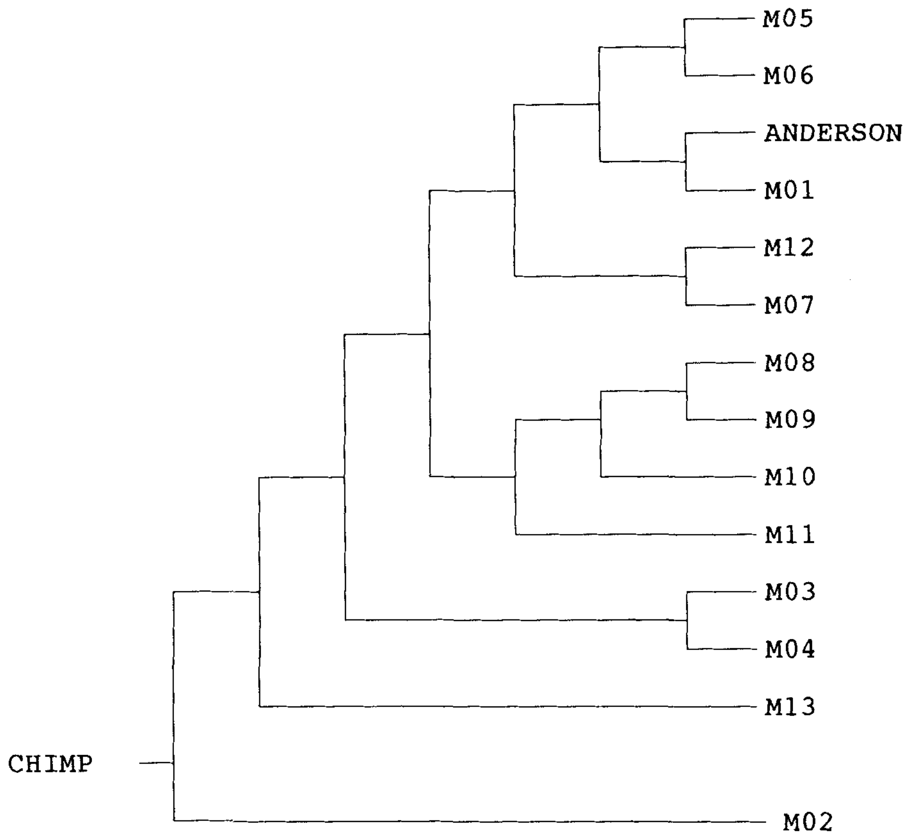

Fig. 2. Consense tree of 13 ancestral mtDNA haplotypes compared to the sequence of chimpanzee and to the sequence of Anderson et al. (1981). The distance matrixes were obtained by the maximum likelihood method on 100 bootstraps of the original values, and the trees were constructed by the method of neighbor-joining. Finally, a consense tree topology was derived from the analysis of the trees obtained.

were combined ("group VI") only to indicate that they did not seem to belong to any of the previous groups. This does not imply that they belong to a single group, because they did not share any mutation that might define them as a homogeneous group. Haplotype M12 differed only in the (C → T) transition in nt 16,223 from the standard sequence of Anderson et al. (1981) used for sequence comparison. This mutation was also present in haplotype M11 together with transition (C → T) in nt 16,111. Haplotype M13 had the (T → C) transition in nt 16,311 and in nt 16,362.

Finally, five different sequences were detected in the hypervariable segment of the D-loop region (Table 3): in addition to the standard sequence (Anderson et al., 1981), a T → C transition was observed in nt 16,189 in three specimens, and sample 11 we also detected two A → – deletions in nt 16,182 and nt 16,183, respectively, and two – → C insertions in nt 16,183.3 and nt 16,183.4, respectively, which represent other characteristics of haplogroup I.

DISCUSSION

We were able to sequence the D-loop region of mtDNA from 18 bone samples from native Indians of the Brazilian Amazon region covering a period of approximately 3,500 years immediately preceding the beginning of the European colonization. De-

TABLE 3. mtDNA sequences in the hypervariable domain observed in our samples of ancestral DNA and its distribution in the major ethnic groups as described by Horai et al. (1993)

No.	mtDNA sequences	Europeans	Asians	Amerindians
1	AAAA----CCCCCTCCCC[1]	15	55	36
2	AAAA----CCCCCCCCCC	01	07	04
3	AAAA----CCCCCCCCTC	—	—	—
4	AAAA----CCCCCTCCTC	—	01	02
5	AA----CCCCCCCCCCCC	—	—	05

[1] Sequence described by Anderson et al. (1981).

spite the fact that this was a relatively small sample, a wide haplotype variability was demonstrable: in addition to the four haplogroups described by Horai et al. (1993) corresponding to 61% of the sample, there were eight samples that did not belong to any of these haplogroups, which we have tentatively assembled into groups V and VI.

The assignment of the observed haplotypes to the four sequence haplogroups described by Horai et al. deserve some comments. Haplogroup II has no dignostic marker that is not shared with any other group: it is characterized by three transitions (at nt 16,223, nt 16,325, and nt 16,362), all of which must be present. Our haplotype M02 had the first two changes and, although the sequencing did not reach the third point, it is reasonable to assume that it belongs to this haplogroup. The assignment of haplotypes M05, M06, and M07 to group IV was based on the presence of only one of the diagnostic mutations (the T → C transition at nt 16,298 for M05 and M06; the C → T at nt 16,327 for M07) of the haplogroup, and also because these three haplotypes clustered with other haplotypes of this haplogroup when compared to sequences of contemporary Amerindians. We believe that this conservative interpretation is appropriate because of the small number of ancestral sequences which are still available.

Haplogroup V was more homogeneous than our group VI, and the haplotypes shared the (C → T) transition in nt 16,223 and nt 16,278. The two mutations are shared by different ethnic groups, indicating that they precede by a long time the entry of the first inhabitants into the Americas. The (C → T) transition in nt 16,233 is detected at low frequency among Blacks, Caucasians, and Mongoloids of the Asia-I group, and at high frequency among Mongoloids of the Asia-II group (Horai and Hayasaka, 1990). Among Amerindians, this (C → T) transition is frequently associated with haplotypes of groups II–IV. The (C → T) transition in nt 16,278 is also detected in Black and Caucasian populations. In Asian populations, the transition is associated with haplogroup I in Asia-I and haplogroup II in Asia-II. A haplotype of the New Guinea population has the mutation and does not fit any of the I–IV haplogroups. Among Amerindians, the mutation has been detected in the Pima tribe (USA) in association with haplogroup I and in the Krahó tribe (Brazil) in association with haplogroup III (Torroni et al., 1993a) and Nuu-Chah-Nulth (USA) (Ward et al., 1991). In the last tribe, four haplotypes similar to those defined as group V have been described in seven individuals (10% of the total sample).

Our assignment of three haplotypes to group VI does not indicate that they belong to a single haplogroup; rather, it indicates that they cannot be included in the other five groups. These haplotypes are heterogeneous but they have at least one mutation typically detected in Asian and Mongoloid populations. The mutation at point 16,362 (T → C) is also present in different ethnic groups (Blacks, Caucasians, and Mongols) in association with haplogroups I–IV (Horai and Hayasaka, 1990; Ward et al., 1991; Torroni et al., 1993b). Mutation at point 16,111 (C → T) probably arose in a Siberian subpopulation that gave origin to Amerindians and Nadene.

The possibility that the haplotypes of groups V and VI were from skeletons of other racial groups can be ruled out because: (1) the pattern of burial in fetal position in ceramic anthropomorphic urns or in sambaquis is typically indigenous, and (2) all the haplotypes present at least one mutation

characteristic of Asian populations or groups derived from Asia. The possibility of contamination with exogenous DNA during laboratory processing can also be ruled out. In addition to all the precautions taken (see Material and Methods), it is significant that all of the 13 haplotypes observed were different from those of the researchers directly involved in handling the samples in the laboratory, and only three (M01–M03) were identified in samples previously sequenced in our laboratory. The amplification of mtDNA segments in the range of 500 bp obtained from ancient Amerindian material has also been previously reported (Monsalve et al., 1994).

If our sample is representative of Pre-Columbian America, the proportion of haplotypes not belonging to the four haplogroups of Horai et al. (1993) is much greater than in contemporary indigenous populations. This finding, however, is also supported by our results obtained in contemporary Amerindian populations from the Amazon, showing that 7% of the haplotypes obtained concomitantly by RFLP and sequencing lack one of the characteristic markers of the four haplogroups (S.E.B. Santos, A.K.C. Ribeiro-dos-Santos, D. Meyer, and M.A. Zago, unpublished). This permits us to suggest that the reduction in genetic variability observed in present-day Amerindian populations could also be attributed to the depopulation effect that started in the 16th century, which decreased the population size by more than 95% (Dobyns, 1966), in addition to the bottleneck effect during migration from Asia to the Americas, as proposed earlier.

Finally, we point out the absence of mutations characteristic of populations from Oceania. This finding, however, has little effect on the arguments about contributions by the Pacific Ocean people to the origin of Amerindians.

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